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The binding of Mg(II) and Ni(II) to synthetic polynucleotides

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Equilibria and kinetics of the interactions of Mg^{2+} and Ni^{2+} with poly(U), poly(C) and poly(I) have been investigated at 25 °C, an ionic strength of 0.1 M, and pH 7.0 or 6.0. Analogous studies involving poly(A) were reported earlier. All binding equilibria were studied by means of the (usually small) absorbance changes in the ultraviolet range. This technique yields apparent binding constants which are fairly large for the interaction of Ni^{2+} with poly(A) ($K = 0.9 \times 10^4$ M^{-1}) and poly(I) ($K \approx 2 \times 10^4$ M^{-1}) but considerably lower for the corresponding Mg^{2+} systems, Mg^{2+}-poly(A) ($K = 2 \times 10^3$ M^{-1}) and Mg^{2+}-poly(I) ($K = 280$ M^{-1}). Each of the two pyrimidine nucleotides binds both metal ions with about the same strength ($K \approx 65$ M^{-1} for poly(U) and K near 600 M^{-1} for poly(C)). In the case of poly(C) the spectral changes deviate from those expected for a simple binding equilibrium. In addition, the binding of Ni^{2+} to the four polynucleotides was measured by using murexide as an indicator of the concentration of free Ni^{2+}. The results obtained by this technique agree or are at least consistent with those derived from the ultraviolet spectra. Complications are encountered in the binding studies involving poly(I), particularly at higher metal ion concentrations, obviously due to the formation of aggregated poly(I) species. Kinetic studies of the binding processes were carried out by the temperature-jump relaxation technique. Measurable relaxation effects of time constants greater than 5 μs were observed only in the systems Ni^{2+}-poly(A) and Ni^{2+}-poly(I). Such not-too-fast reaction effects are expected for processes which include inner-sphere substitution steps at Mg^{2+} or Ni^{2+}. The relaxation process in Ni^{2+}-poly(I) is characterized by (at least) four time constants. Obviously, the complicated kinetics again include reactions of aggregated poly(I). The absence of detectable relaxation effects in all other systems (except Mg^{2+}-poly(I), the kinetics of which was not investigated) indicates that inner-sphere coordination of the metal ions to specific sites of the polynucleotides (site binding) does not occur to a significant extent. Rather, the metal ions are bound in these systems mainly by electrostatic forces, forming a mobile cloud. The differences in binding strength which are nevertheless observed are attributed to differences in the conformation of the polynucleotides which result in different charge densities.

1. Introduction

Divalent metal ions participate in nearly all biological processes involving nucleotides. They activate mononucleotides in phosphoryl transfer reactions [1], stabilize the double-helical structure of DNA [2,3] and are needed to preserve the specific conformation of tRNA [2,3]. Divalent metal ions are also required in the various steps of genetic information transfer, i.e., replication of DNA, transcription of DNA to RNA, and protein synthesis at the ribosomes [2]. Mg^{2+} is of particular importance in all these processes. In vitro, however, Mg^{2+} can often be replaced by other metal ions, e.g., Mn^{2+}, Co^{2+} and Zn^{2+} [2]. There are indications that the interactions of transition metal ions and Zn^{2+} with nucleotides are also of relevance for biological systems: Small amounts of a variety of transition metal ions have been found in RNAs from many different sources (after extended dialysis against complexing agents) [4] and it is assumed that they too help to stabilize a specific polynucleotide conformation [4]. More re-

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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cently, it has been established that several DNA and RNA polymerases are zinc enzymes [5] and that the metal center participates in the catalytic action [6].

Because of their biological importance, metal ion-nucleotide interactions in solution have been studied extensively and the results have been repeatedly reviewed [7]. Previous publications from this laboratory reported on the equilibria and kinetics of the binding of transition metal ions (Ni^{2+}, Co^{2+}) to several nucleosides and mononucleotides [8] and also to poly(A) [9]. In the present paper we report on measurements of the equilibria and kinetics for the binding of Mg^{2+} and Ni^{2+} to the synthetic homopolynucleotides poly(U), poly(C) and poly(I) under near-physiological conditions with respect to ionic strength and pH. Ni^{2+}-nucleotides are not particularly important in biology; Ni^{2+} is just considered to be a representative member of the first-row transition metals. In addition, complex formation reactions of Ni^{2+} are slower than those of other divalent transition metal ions (except V^{2+}) and therefore their kinetics are more conveniently measured.

2. Materials and methods

Poly(U), poly(C) and poly(I) were obtained from P-L Biochemicals. Stock solutions of the polynucleotides (4×10^{-4}–1.5×10^{-2} M in monomeric units) were kept in the dark at 0 °C and used within 8 days. The polynucleotide solutions were standardized spectrophotometrically, using the extinction coefficients given in the 'Certificate of Analysis'. Nickel perchlorate (Fluka), nickel nitrate (Merck), magnesium perchlorate (Merck), tetramethylammonium chloride (Schuchardt), murexide (Merck), sodium cacodylate (Fluka) and other common chemicals were of the best grade commercially available and were used without further purification. The stock solutions of Mg^{2+} and Ni^{2+} were standardized by complexometric titrations [10]. All solutions were prepared with water doubly distilled from a quartz apparatus.

Measurements were carried out at 25.0 (±0.1) °C, ionic strength $I = 0.1$ M (during some of the titrations the ionic strength increased to about 0.3 M, see below), pH 7.0 or 6.0. Measurements of pH were made using a Radiometer PHM 52 digital pH-meter equipped with a Metrohm EA125 combined electrode. For measurements in solutions containing perchlorate, the KCl solution in the reference compartment of the electrode was replaced by a NaCl solution. The pH-meter was calibrated using Merck Titrisol buffer solutions. The pH of all reactant solutions was stabilized by adding 1×10^{-3} M cacodylate buffer. At this concentration the buffer does not interact noticeably with divalent metal ions [8a].

The binding equilibria were studied spectrophotometrically, using a Cary 118 spectrophotometer. The polynucleotides absorb in the ultraviolet ($\lambda_{max} = 248$–268 nm, $\varepsilon_{max} = 6.5 \times 10^3$–$10.2 \times 10^3$), due to the π-π^* and n-π^* transitions of the nucleotide bases. The spectral changes which are observed upon the addition of divalent metal ions were recorded. Since these changes are small, difference measurements at a high sensitivity of the instrument (0.1 or 0.2 absorbance units/full scale) were carried out. Using two-compartment cells (path length of each compartment 1 cm), the difference (poly(N) + M^{2+})(H_2O) vs. (poly(N)) (M^{2+}) was recorded, where N = C, U or I, and $M^{2+} = Mg^{2+}$ or Ni^{2+}. All compartments contained 0.1 M of inert salt (ionic strength adjustment) and 1×10^{-3} M cacodylate. In order to keep dilution effects low, small amounts (up to a total of 200 μl at most) of concentrated M^{2+} solutions (0.01–0.98 M) were added to 2.5 ml of the nucleotide solution (4×10^{-5}–1.1×10^{-4} M) in the sample pathway and to the nucleotide-free compartment in the reference pathway by means of a micrometer syringe unit. The dilution of the nucleotide solution in the sample pathway was compensated by adding the same volume of 0.1 M inert salt solution to the nucleotide solution in the reference pathway.

Since the ultraviolet spectral changes which are observed during the titrations reflect not only direct metal ion-base interactions but also changes in the degree of base stacking due to metal ion binding, and since these two contributions cannot be distinguished, a second method was applied to evaluate quantitatively the binding of Ni^{2+} to the polynucleotides. This method uses murexide as an indicator of the concentration of free Ni^{2+} and has been described previously [9]. The apparent stability constant of the Ni^{2+}-murexide complex is close to 1×10^4 M^{-1} at pH 7.0 and $I = 0.1$ M [9]. Therefore, concentrations of free Ni^{2+} from about 10^{-5} to 10^{-3} M can be determined by this technique. The large spectral changes near 520 nm which are produced by the binding of Ni^{2+} to murexide are illustrated in fig. 1.

The kinetic measurements were carried out by means of the temperature-jump relaxation technique with spectrophotometric detection [11]. The relaxation processes were observed in the ultraviolet (no murexide present). The reactant solution in the cell was thermostatted at 22.0°C before raising the temperature to 25.0°C by discharging a 0.05 μF capacitor of 30 kV. The relaxation signal was stored in a Biomation 1010 transient recorder. This recorder enables the registration of the relaxation process with two different time bases in one experiment, thus covering a wide time range. Each signal was displayed on a Hewlett-Packard 1220A oscilloscope screen and – if not visibly disturbed – transferred via a PDP 11/34 to a Univac 1108 computer. Since the signal/noise ratio was not good in the kinetic studies (small relaxation amplitudes), about 10 individual signals of each solution obtained under identical conditions were sampled, averaged and evaluated by the Univac computer. The evaluation was done according to the method of Provencher [12].

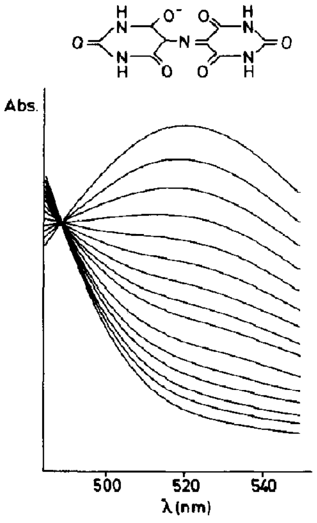

Fig. 1. Spectra of murexide before (upper curve) and after addition of increasing amounts of Ni^{2+} (lower curves).

3. Results

3.1. Metal ion binding equilibria

3.1.1. Poly(A)

For comparison and completeness the experimental results involving poly(A) are included in this section.

3.1.1.1. Ni^{2+} binding. In an earlier study it was shown by applying the murexide technique that Ni^{2+} binds strongly to single-stranded poly(A) in 0.1 M KNO_3 at pH 7.0 [9]. Within the accessible concentration range the data yielded a linear Scatchard plot from which the apparent number of binding sites per monomer was obtained (m = 0.26) and the stability constant for binding of Ni^{2+} to these sites ($K = 8.2 \times 10^3$ M^{-1}) [9].

The ultraviolet spectral changes which occur during a titration of poly(A) with Ni^{2+} have also been recorded. To 3 ml of a 7.6×10^{-5} M poly(A) solution (0.1 M KNO_3, pH 7.0) were added 5-μl portions of 0.025 M Ni^{2+}, up to a total of 25 μl, and – to a new poly(A) solution – 5-μl portions of 0.098 M Ni^{2+}, up to a total of 30 μl. After each addition the difference spectrum was measured. Good isosbestic points are observed at 276 and near 225 nm. The difference spectrum has a maximum at 253 nm. The spectral changes at 253 nm as a function of the total Ni^{2+} concentration are shown in fig. 2 (full circles). The experimental data are consistent with a simple binding equilibrium of the form

$$K = [NiS]/[Ni^{2+}][S] \qquad (1)$$

where S represents a binding site. With $[NiS] + [S] = [S]_0$ = total concentration of binding sites, $[NiS] + [Ni^{2+}] = [Ni^{2+}]_0$ = total Ni^{2+} concentration, $[NiS] = (A - A_0)/(\varepsilon_{NiS} - \varepsilon_S) = \Delta A/\Delta\varepsilon$ and $[S]_0 = (A_\infty - A_0)/\Delta\varepsilon = \Delta A_\infty/\Delta\varepsilon$ (ε, extinction coefficient), where A denotes the absorbancy per cm path length, A_0 its initial value ($[Ni^{2+}]_0 = 0$) and

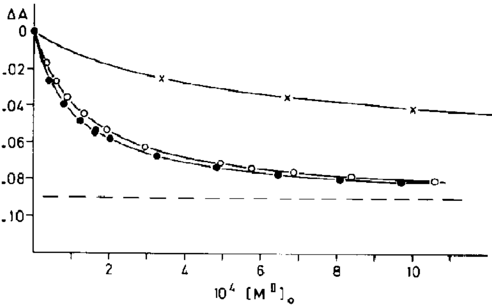

Fig. 2. Spectral changes of poly(A) at 253 nm as a function of total metal ion concentration. (●) Addition of Ni^{2+}, (○) points calculated for the addition of Ni^{2+} using m and K from the murexide studies, (×) addition of Mg^{2+} (25 °C, 0.1 M KNO_3, pH 7.0; M.J. Hynes and H. Diebler, unpublished data).

A_∞ its limiting value at very high concentrations of Ni^{2+}, one obtains from eq. 1 the expression

$$\frac{\Delta A_\infty}{\Delta A_\infty - \Delta A} = K \cdot \frac{\Delta A_\infty}{\Delta A}[Ni^{2+}]_0 - K[S]_0. \tag{2}$$

A plot according to eq. 2 is shown in fig. 3. The slope of this plot gives $K = 9.1\ (\pm 0.2) \times 10^3\ M^{-1}$ (25 °C, $I = 0.1$ M, pH 7.0). The intercept is indistinguishable from zero. This means that at the low poly(A) concentrations used in the ultraviolet spectrophotometric titrations the concentration of binding sites, $[S]_0$, cannot be determined. The value of ΔA_∞ required for this evaluation was obtained from a plot of $[Ni^{2+}]_0/\Delta A$ vs. $[Ni^{2+}]_0$. Applying the Benesi-Hildebrand approximation [13], $[NiS]^2 \ll [Ni^{2+}]_0[S]_0$, which is justified under the given conditions, such a plot should give a straight line (as indeed was found but is not shown here) with slope $1/[S]_0 \cdot \Delta\varepsilon = 1/\Delta A_\infty$. The value obtained, $\Delta A_\infty = 0.090$, is given in fig. 2 by the horizontal broken line.

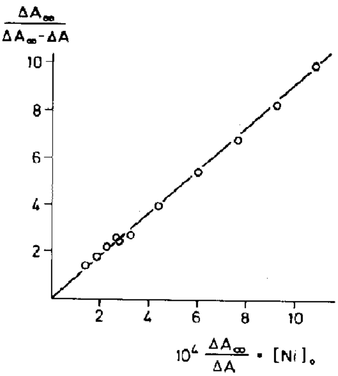

Fig. 3. Ni^{2+}-poly(A). Plot of $\Delta A_\infty/(\Delta A_\infty - \Delta A)$ vs. $(\Delta A_\infty/\Delta A) \cdot [Ni^{2+}]_0$ according to eq. 2.

The open circles in fig. 2 represent points which have been calculated for the same change in absorbancy with the data obtained by the murexide technique, $m = 0.26$ and $K = 8.2 \times 10^3\ M^{-1}$.

3.1.1.2. Mg^{2+} binding. The results of a titration of 8.4×10^{-5} M poly(A) with 0.20 M Mg^{2+} are denoted by the crosses in fig. 2. The data again conform to a simple binding equilibrium. The evaluation (as before) yields $K = 2.0 \times 10^3\ M^{-1}$ (25 °C, $I = 0.1$ M KNO_3, pH 7.0). Here, too, the value of $[S]_0$ cannot be determined.

3.1.2. Poly(U)

3.1.2.1. Ni^{2+} binding. For the reasons discussed before [9], measurements of the binding of Ni^{2+} to the polynucleotides by the murexide technique have been carried out in the presence of a potassium salt (KNO_3) as inert electrolyte. Preliminary experiments with poly(U) indicated only weak Ni^{2+} binding. In order to decrease the uncertainty in the amount of Ni^{2+} bound to the polymer, high concentrations of poly(U) (about 1.3×10^{-2} M) had to be used in these studies. To 3 ml of a solution containing poly(U) and murexide were added microliter amounts of 0.082 M Ni^{2+}, up to a total of 50 μl. After each addition the spectrum was recorded. The dilution effects, though small, were corrected for in the evaluation. The concentration of Ni^{2+} bound to the polymer was calculated from [9]

$$[Ni^{2+}_{bound}] = [Ni^{2+}]_0 - [Ni^{2+}_{free}] - [NiMur] \tag{3}$$

where

$$[Ni^{2+}_{free}] = \frac{1}{K_{app}} \cdot \frac{A_0 - A}{A - A_\infty} \tag{4}$$

and

$$[NiMur] = \frac{A_0 - A}{A_0 - A_\infty}[Mur]_0. \tag{5}$$

Here, A represents the absorbance due to the murexide species (measured at 521 nm) at a given Ni^{2+} concentration, A_0 its initial value (absence of Ni^{2+}), and A_∞ the value if all of the murexide has been converted into NiMur. $K_{\mathrm{app}} = 1.03 \times 10^4$ M^{-1} denotes the apparent stability constant of the NiMur complex at 25°C, $I = 0.1$ M and pH 7.0 [9].

The number n of Ni^{2+} bound per monomeric unit of poly(U) is then given by

$$n = [\mathrm{Ni}^{2+}_{\mathrm{bound}}]/[\mathrm{poly(U)}] \qquad (6)$$

where [poly(U)] denotes the concentration of poly(U) expressed in monomeric units (as is done throughout this paper). Values of $n/[\mathrm{Ni}^{2+}_{\mathrm{free}}]$ vs. n can now be plotted (Scatchard plot). The results for Ni^{2+}-poly(U) are shown in fig. 4. The low values of n and $n/[\mathrm{Ni}^{2+}_{\mathrm{free}}]$ indeed confirm that poly(U) binds Ni^{2+} only very weakly. At concentrations of $\mathrm{Ni}^{2+}_{\mathrm{free}}$ close to 1×10^{-3} M (the upper limit for the murexide method) the value of n is still below 0.02, i.e., less than 1 Ni^{2+} is bound per 50 monomeric units. Correspondingly low are the values of $n/[\mathrm{Ni}^{2+}_{\mathrm{free}}]$, near 30 M^{-1}. Moreover, this quantity shows little variation with the concentration of Ni^{2+} (within the accessible concentration range). The experimental points (fig. 4) do not form a straight line with well defined intercept and slope, and the Scatchard parameters m (number of binding sites per monomer) and K cannot be evaluated.

In such cases of weak metal ion binding, ultraviolet difference spectrophotometry offers the advantage that measurements can be extended to much higher metal ion concentrations. A 1.1×10^{-4} M solution of poly(U) was titrated with 0.98 M Ni^{2+}, up to a concentration of 0.075 M Ni^{2+} in poly(U). During the titration the ionic strength increased from 0.1 to about 0.3 M. Because of the ultraviolet absorption of NO_3^-, a perchlorate medium (NaClO_4, $\mathrm{Ni(ClO_4)_2}$) was chosen for these studies. In order to reduce the hydrolysis of Ni^{2+} and to avoid the formation of colloidal or precipitating Ni-hydroxide species at high Ni^{2+} concentration, the measurements were carried out at pH 6.0 instead of 7.0. Otherwise the same procedure as outlined above was employed. The results are represented by the open circles of fig. 5

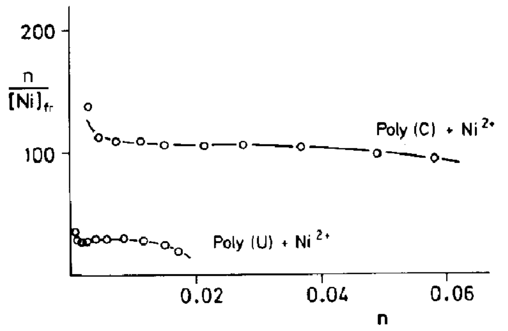

Fig. 4. Scatchard plots ($n/[\mathrm{Ni}^{2+}_{\mathrm{free}}]$ vs. n) for Ni^{2+}-poly(U) and Ni^{2+}-poly(C) (25°C, 0.1 M $\mathrm{KNO_3}$, pH 7.0).

If the binding of Ni^{2+} to poly(U) can be described by a simple equilibrium as formulated by eq. 1, then the experimental data should conform to the relationship

$$\frac{[\mathrm{Ni}^{2+}]_0}{\Delta A} = \frac{1}{K} \cdot \frac{1}{\Delta A_\infty} + \frac{[\mathrm{Ni}^{2+}]_0}{\Delta A_\infty}. \qquad (7)$$

Eq. 7 has been derived applying the Benesi-Hildebrand approximation [13]. This approximation is fully justified, since always $[\mathrm{Ni}^{2+}]_0 \gg [\mathrm{S}]_0$ under the conditions of measurement. Plots of $[\mathrm{Ni}^{2+}]_0/\Delta A$ vs. $[\mathrm{Ni}^{2+}]_0$ indeed yielded straight lines (not shown). The ratio slope/intercept of the plots of two runs gave for Ni^{2+}-poly(U) $K = 67$ (± 4) M^{-1} (25°C, $I = 0.1$–0.3 M, pH 6.0).

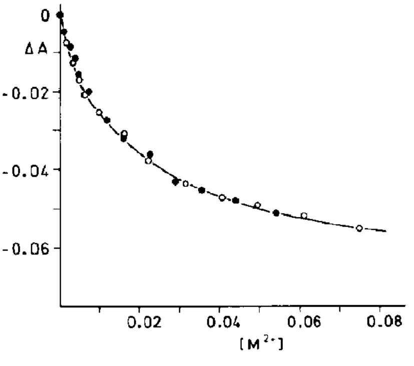

Fig. 5. Spectral changes of poly(U) at 257 nm as a function of total metal ion concentration. (○) Addition of Ni^{2+}, (●) addition of Mg^{2+} (25°C, $I = 0.1$–0.3 M (perchlorate), pH 6.0).

3.1.2.2. Mg^{2+} binding. A titration of a 1.1×10^{-4} M solution of poly(U) with 0.70 M Mg^{2+} was carried out as described in section 3.1.2.1. The experimental data are included in fig. 5 (full circles). They are almost indistinguishable from those obtained with Ni^{2+}. A plot of the data for Mg^{2+}-poly(U) according to eq. 7 is shown in fig. 6. The slope and intercept of the straight line of fig. 6 yield for Mg^{2+}-poly(U) $K = 63$ M^{-1} (25 °C, $I = 0.1$–0.25 M, pH 6.0).

3.1.3. Poly(C)

3.1.3.1. Ni^{2+} binding. This polynucleotide also binds Ni^{2+} ions rather weakly, though somewhat more strongly than poly(U). Measurements by the murexide method in 1.0×10^{-2} M poly(C) solution provided the data displayed in fig. 4 (upper series). The values of $n/[Ni^{2+}_{free}]$ are near 100 and do not vary much with the Ni^{2+} concentration. At concentrations of free Ni^{2+} close to 1×10^{-3} M (upper limit) the values of n are still below 0.1, i.e., less than 1 Ni^{2+} is bound per 10 monomeric units.

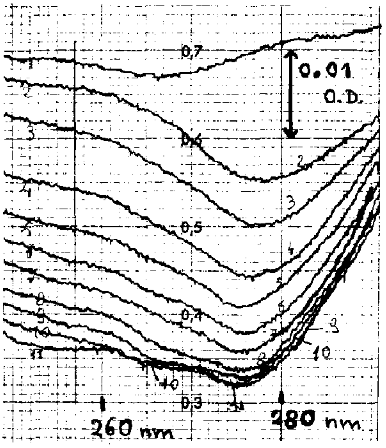

Fig. 7. Difference spectra Ni-poly(C) vs. poly(C). $[Poly(C)]_0 = 8.8 \times 10^{-5}$ M; $[Ni^{2+}]_0 = 0$–0.07 M. Upper trace: baseline ($[Ni^{2+}]_0 = 0$) (25 °C, $I = 0.1$–0.3 M (perchlorate), pH 6.0).

The ultraviolet difference spectra obtained by titrating an 8.9×10^{-5} M solution of poly(C) with 0.98 M Ni^{2+} are shown in fig. 7. They reveal that the spectral changes (which are very small) cannot be accounted for by only one binding equilibrium. Plots of ΔA vs. $[Ni^{2+}]_0$ for the data at the wavelengths 257 and 280 nm illustrate this complex behavior (see fig. 8). Actually, the data at 280 nm are consistent with just one binding equilibrium, and a plot according to eq. 7 (not shown) leads to a good straight line from which an apparent equilibrium constant $K = 660$ (± 100) M^{-1} is obtained (small intercept, therefore large uncertainty). By contrast, the data for 257 nm do not fit eq. 7. If the first two points are neglected, the spectral changes at 257 nm lead to an approximate apparent binding constant of 220 M^{-1}.

3.1.3.2. Mg^{2+} binding. Addition of Mg^{2+} to a solution of poly(C) produces changes in the ultraviolet spectrum which are similar to those observed with Ni^{2+}. The spectral changes at 280 nm

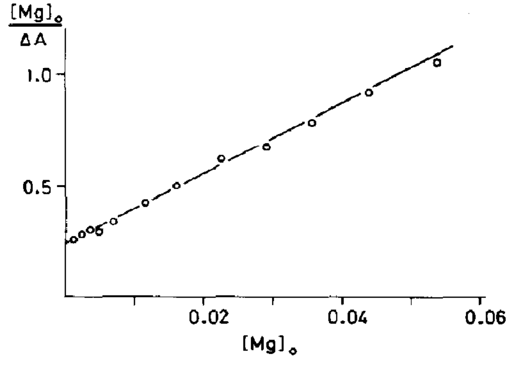

Fig. 6. Mg^{2+}-poly(U). Plot of $[Mg^{2+}]_0/\Delta A$ vs. $[Mg^{2+}]_0$ according to eq. 7 (25 °C, $I = 0.1$–0.25 M, pH 6.0).

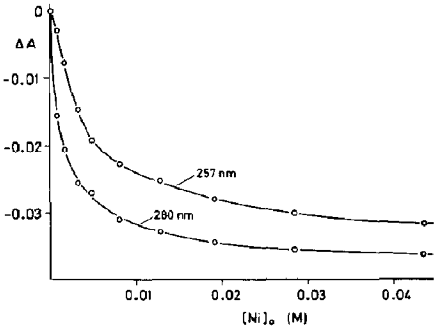

Fig. 8. Ni^{2+}-poly(C). Spectral changes at 257 and 280 nm as a function of total Ni^{2+} concentration (25 °C, $I = 0.1$–0.23 M (perchlorate), pH 6.0).

can approximately be described by a single binding process with an equilibrium constant $K = 530$ (± 50) M^{-1} (25 °C, $I = 0.1$–0.23 M (perchlorate), pH 6.0). At 257 nm the plot of ΔA vs. $[Mg^{2+}]_0$ begins with a slightly sigmoidal section, as in the case of Ni^{2+} (see fig. 8). If the first two points for 257 nm are neglected, the remaining data for this wavelength can again be fitted roughly to a simple binding equilibrium; the apparent stability constant is near 190 M^{-1}.

3.1.4. Poly(I)

3.1.4.1. Ni^{2+} binding. Preliminary studies with this polynucleotide indicated strong Ni^{2+} binding. Using murexide in order to determine the concentration of free Ni^{2+}, a 1.5×10^{-3} M solution of poly(I) in 0.1 M KNO_3 was titrated with 0.082 M Ni^{2+} at pH 7.0. The data obtained lead to the Scatchard-type plot represented by the open circles of fig. 9. At a concentration of free Ni^{2+} of 0.3×10^{-3} M, the value of n is already 0.50, i.e., 1 Ni^{2+} is bound per 2 monomeric units. Correspondingly high are the values of $n/[Ni^{2+}_{free}]$. This Scatchard plot is not at all linear; after passing through a flat minimum the values of $n/[Ni^{2+}_{free}]$ increase again somewhat between $n = 0.3$ and 0.5. In this region the relative increase in bound Ni^{2+} is larger than that in free Ni^{2+}. Presumably, this peculiar behavior is a consequence of conformational transitions of poly(I). In particular, it is well known that poly(I) has a strong tendency to aggregate under formation of four-stranded helices in the presence of metal ions such as Na^+ and K^+ [14]. Therefore, in order to reduce aggregation, additional experiments using the murexide technique have been carried out in 0.1 M tetramethylammonium chloride (TMACl) as inert electrolyte instead of 0.1 M KNO_3 and at lower poly(I) concentrations (2.3×10^{-4} M). The Scatchard-type plot obtained under these conditions is also shown in fig. 9, represented by the full circles. (The stability constant of the Ni-Mur complex was redetermined in 0.1 M TMACl; a slightly higher value than in KNO_3 was obtained, $K_{app} = 1.2 \times 10^4$ M^{-1} (pH 7.0), and used in the evaluation.) The values of n and $n/[Ni^{2+}_{free}]$, less accurate at the lower poly(I) concentration, are of the same magnitude as before. There is an approximately linear section in the Scatchard plot (fig. 9), but at higher Ni^{2+} concentrations strong deviations from linearity are found, most likely due to aggregation induced by Ni^{2+}. At still higher concentrations (starting around 0.8×10^{-3} M $[Ni^{2+}]_0$) the murexide absorption bands do not meet the isosbestic point, and after standing for several hours a precipitate is observed.

The results of ultraviolet difference measurements at 250 nm are shown in fig. 10. The open circles refer to a titration of 8.3×10^{-5} M poly(I) with 0.99 M Ni^{2+} (in 0.1 M TMACl, pH 7.0), and the full circles to one of 4.15×10^{-5} M poly(I) with 0.01 M Ni^{2+} (0.1 M TMACl, pH 7.0). The values of ΔA at 250 nm do not reach a limiting value upon further addition of Ni^{2+}, but the dif-

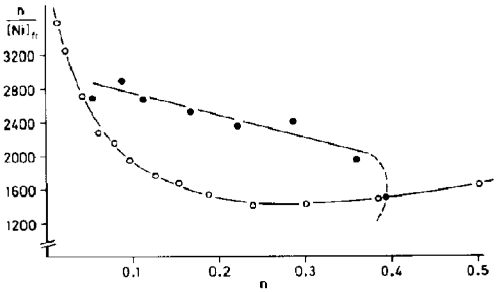

Fig. 9. Scatchard plots for Ni^{2+}-poly(I). (○) 1.5×10^{-3} M poly(I) in 0.1 M KNO_3, pH 7.0; (●) 2.3×10^{-4} M poly(I) in 0.1 M TMACl, pH 7.0.

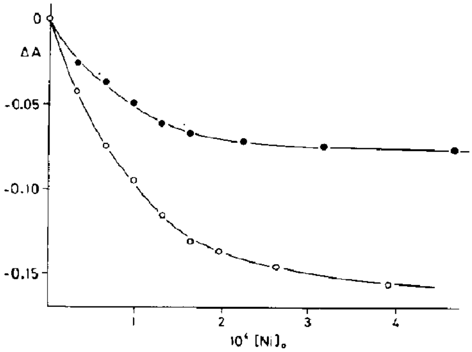

Fig. 10. Ni^{2+}-poly(I). Spectral changes at 250 nm as a function of total Ni^{2+} concentration. (○) 8.3×10^{-5} M poly(I); (●) 4.15×10^{-5} M poly(I) (25 °C, 0.1 M TMACl, pH 7.0).

ference bands begin to change their structure and shape (starting around 0.5×10^{-3} M $[\mathrm{Ni}]_0$) and the (absolute) value of ΔA_{250} even decreases again. This behaviour presumably reflects aggregation induced by Ni^{2+}. After standing for several hours, a precipitate is observed. Because of these complications, exact binding constants cannot be evaluated from the data shown in fig. 10, particularly since the Benesi-Hildebrand approximation cannot be applied (strong binding). However, an approximately linear relationship is found if the data of fig. 10 (open circles) are plotted according to eq. 2 with $\Delta A_\infty = 0.18$ (fig. 11). The slope of this plot yields $K = 2.1 \times 10^4$ M^{-1}, and from the intercept is obtained $[S]_0 = 7.3 \times 10^{-5}$ M. Thus, $[S]_0$ is close to $[\text{poly(I)}]_0$ (8.3×10^{-5} M), and this is consistent with the murexide studies which showed that n (number of Ni^{2+} per monomer) can reach and exceed 0.5. We conclude, therefore, that within the concentration range of fig. 11 the ultraviolet data indicate an apparent stability constant for the binding of Ni^{2+} to poly(I) which is close to 2×10^4 M^{-1} (25 °C, 0.1 M TMACl, pH 7.0).

3.1.4.2. Mg^{2+} binding. The addition of Mg^{2+} to poly(I) also leads to changes in the ultraviolet absorption, with a maximum at 250 nm. As compared to Ni^{2+}, much higher concentrations of metal ion are required in order to achieve similar changes in absorbancy. A poly(I) solution of concentration 8.3×10^{-5} M in 0.1 M TMACl, pH 7.0, was titrated with 0.07 M Mg^{2+}. The results are given in fig. 12 in form of a plot according to eq. 7. At Mg^{2+} concentrations above 4.5×10^{-3} M, the difference bands change their shape, obviously because of conformational transition. The slope and intercept of the plot shown in fig. 12 yield for the stability constant of Mg^{2+}-poly(I): $K = 280$ M^{-1} (25 °C, 0.1 M TMACl, pH 7.0).

3.2. Kinetics

3.2.1. M^{2+}-poly(A)

The kinetics of the interaction of Ni^{2+} with poly(A) have been reported before [9]. Except for a very fast initial change in absorbancy which occurs within the heating time (~ 4 μs) and which is due to the rapid helix-coil transition of single-stranded poly(A) (also observed in the absence of divalent metal ions), the relaxation effect is composed of two contributions. Computer analysis of the signals established that the faster effect has a time constant of 0.4 ms, independent of the Ni^{2+} concentration. The relaxation time of the slower effect varies between 2.2 and 5.0 ms, exhibiting a characteristic concentration dependence which is shown in fig. 5 of ref. 9. The simplest mechanism which is consistent with the experimental data is described by the following sequence, $Ni^{2+} + S \rightleftharpoons Ni,S \rightleftharpoons NiS \rightleftharpoons NiS' \rightleftharpoons NiS''$, a very fast second-order association followed by three first-order steps. The numerical values which have been determined for some of the equilibrium and rate constants appear reasonable, by and large,

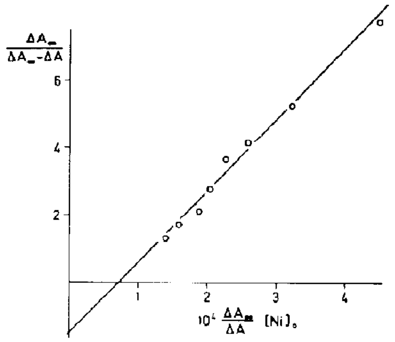

Fig. 11. Ni^{2+}-poly(I). Plot of the data represented by the open circles of fig. 10 according to eq. 2, with $\Delta A_\infty = 0.18$.

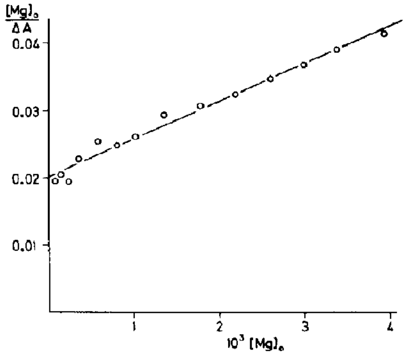

Fig. 12. Mg^{2+}-poly(I). Plot of the spectral changes according to eq. 7 (25 °C, 0.1 M TMACl, pH 7.0, λ = 250 nm).

for the reaction of Ni^{2+} with a polyanion [9].

The kinetics of the binding of Mg^{2+} to poly(A) was studied by Pörschke [15], using the field-jump relaxation technique. At low concentrations of buffer and reactants the relaxation time due to Mg^{2+} binding is in the microsecond range. The second-order rate constant for the binding of Mg^{2+} was evaluated to be $k = 2 \times 10^{10}$ M^{-1} s^{-1}. Because of this very high, close to diffusion-controlled rate, it was concluded that Mg^{2+} does not form inner-sphere complexes with this polynucleotide (site binding) but that it is bound only electrostatically in the form of a mobile cloud. Temperature-jump experiments at higher ionic strength (0.1 M) supported this interpretation [15].

3.2.2. M^{2+}-poly(C) and M^{2+}-poly(U)

Temperature-jump studies of the system Ni^{2+}-poly(C) have been carried out in 0.1 M KNO_3 + 1 $\times 10^{-3}$ M cacodylate, pH 7.0, at 25°C. The poly(C) concentration was 9.9×10^{-5} M, and that of Ni^{2+} was varied from 9×10^{-4} to 1.0×10^{-2} M. At these metal ion concentrations the system is well in the transition range for Ni^{2+} binding (see fig. 8). However, measurable relaxation effects with a time constant greater than 5 μs could not be detected. Only a very fast initial change in light intensity was observed ($\tau < 5$ μs), also in the absence of Ni^{2+}. The failure to detect relaxation processes in the millisecond range apparently indicates that inner-sphere binding of Ni^{2+} to specific sites of poly(C) does not occur to an appreciable extent.

Kinetic studies of the binding of Mg^{2+} to poly(C) have been reported previously [15]. The results are analogous to those obtained for Mg^{2+}-poly(A), discussed above, and indicate that in this system, too, only outer-sphere association is of importance.

Temperature-jump experiments on the system Ni^{2+}-poly(U) were carried out under the same conditions as those described above for Ni^{2+}-poly(C), and the same observations have been made. Evidence for inner-sphere binding could not be detected.

3.2.3. Ni^{2+}-poly(I)

For the reasons given in section 3.1.4, the temperature-jump studies were carried out in 0.1 M TMACl (pH 7.0, 25°C). Preliminary experiments revealed that relaxation processes can be observed in Ni^{2+}-poly(I) solutions. The signal/noise ratio of the effects is most favorable at 265 nm, a wavelength where the xenon/mercury high-pressure lamp has an emission band of high intensity. In most of the experiments the concentration of poly(I) was 2.0×10^{-4} M, and that of Ni^{2+} varied between 2.0×10^{-5} and 4.6×10^{-4} M. At metal ion concentrations above this upper limit, the photomultiplier output signal exhibits erratic fluctuations, obviously due to the formation of colloidal poly(I) aggregates and commencing of precipitation.

The relaxation processes are of a complicated nature; they begin in the microsecond range and extend at the higher Ni^{2+} concentrations to about 1 s. A computer analysis of the relaxation signals revealed that (at least) four time constants are required in order to achieve a reasonable fit to the measured curves. In addition, a very fast change in absorbancy was observed during the heating time (~ 4 μs). For illustration, a relaxation pro-

Table 1

Kinetic data for Ni^{2+}-poly(I)

Conditions: $[\text{poly(I)}]_0 = 2.0 \times 10^{-4}$ M, 25°C, pH 7.0, 0.1 M TMACl, 265 nm. Values in parentheses denote amplitudes (in mV).

$[Ni]_0$ (M)($\times 10^4$)	τ_T (μs)	τ_1 (μs)	τ_2 (ms)	τ_3 (ms)	τ_4 (ms)
4.6	4–5 (0)	~ 200 (1.5)	2.0 (2.0)	14.6 (4.8)	243 (4.3)
3.4	4–5 (9)	~ 100 (2.6)	2.0 (2.1)	16.4 (4.3)	268 (4.5)
2.3	4–5	~ 75	2.1	17.2	278
1.1	4–5 (10)	~ 72 (7.4)	1.3 (6.0)	50.9 (1.1)	–
0.6	4–5 (14)	~ 76 (6.6)	1.1 (4.4)	108 (1.4)	–
0.2	4–5 (14)	~ 50 (6.0)	0.7 (0.9)	–	–

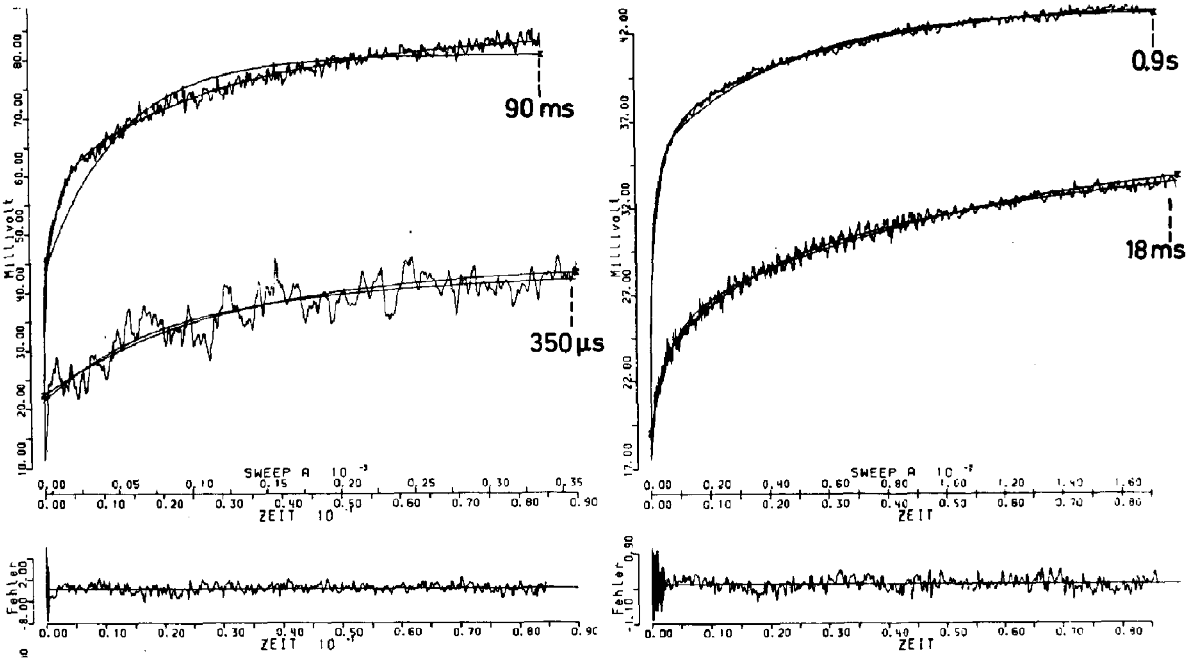

Fig. 13. Relaxation process in Ni^{2+}-poly(I) solution; $[poly(I)]_0 = 2.0 \times 10^{-4}$ M, $[Ni^{2+}]_0 = 2.3 \times 10^{-4}$ M. Two measurements, each one recorded with divided time base. Smooth curves: two fits with different number of time constants (25° C, 0.1 M TMACl, pH 7.0).

cess recorded with different time bases is displayed in fig. 13. Because of the overlap of the various relaxation steps and the small amplitudes (large noise), the time constants could not be evaluated with a high accuracy. A summary of the kinetic data is provided in table 1.

4. Discussion

A survey of the apparent metal ion binding constants evaluated by ultraviolet spectrophotometry during this study is given in table 2.

In general, the ultraviolet spectral changes which are observed upon addition of metal ions may be composed of two contributions: (a) those due to direct metal-ion base interactions, (b) those from changes in conformation. These two contributions do not necessarily exhibit the same dependence on metal ion concentration. It is conceivable that the initial binding of metal ions produces strong conformational changes, whereas that subsequent produces only small or negligible changes in conformation. Therefore, additional studies of Ni^{2+} binding have been carried out in which the amount of Ni^{2+} bound to the polynucleotide was determined by using murexide as an indicator of the concentration of free Ni^{2+}. In the case of poly(A), which exists under our conditions in a largely stacked single-stranded form [16], both techniques yield data which are consistent with a single (noncooperative) equilibrium for Ni^{2+} binding. It thus appears that each Ni^{2+} which is bound produces the same change in ultraviolet absorbancy. Noncooperative binding and linear Scatchard plots have been also reported for several other metal ion-polynucleotide systems [17]. The apparent binding constants for Ni^{2+}-poly(A) which have been obtained by the two techniques differ by only 10%. Kinetic studies demonstrated that inner-sphere complexation is involved in the binding of Ni^{2+} to poly(A) [9]. NMR measure-

Table 2

Apparent metal ion binding constants obtained by ultraviolet spectrophotometry

	K (M^{-1})		Conditions
	Mg^{2+}	Ni^{2+}	
Poly(A) [a]	2.0×10^3	9.1×10^3	25 °C, $I = 0.1$ M, pH 7.0
Poly(I)	280	2×10^4	25 °C, $I = 0.1$ M, pH 7.0
Poly(U)	63	67	25 °C, $I = 0.1$–0.3 M, pH 6.0
Poly(C)	560 [b]	660 [b]	25 °C, $I = 0.1$–0.23 M, pH 6.0

[a] Data by M.J. Hynes and H. Diebler.

[b] From ultraviolet changes at 280 nm.

ments indicate that the phosphate groups [18] and adenine bases [18,19] (in particular the N_7 atom) can be engaged in the site binding of transition metal ions to poly(A). The decrease in absorbancy upon addition of Ni^{2+} to poly(A) (see fig. 2), confirms that at high inert salt concentrations the stacking enhancement due to phosphate binding overcompensates the destabilization of helical structure due to base binding; in the absence of added electrolyte the opposite is observed [20].

In contrast to the transition metal ions, the 'hard' Mg^{2+} shows no tendency for binding to the nucleotide bases [2,7a,21]. It is plausible therefore that the apparent binding constant of Mg^{2+}-poly(A) is about 5-times lower than that for Ni^{2+}-poly(A). Actually, kinetic investigations led to the conclusion that the binding of Mg^{2+} to poly(A) is predominantly electrostatic (non-specific); evidence for inner-sphere binding could not be demonstrated [15]. The observed changes in ultraviolet absorption are then solely due to an Mg^{2+}-induced increase in the degree of base stacking in single-stranded poly(A). Considering the strong ionic-strength dependence of the binding of Mg^{2+} to poly(A), the stability constant reported here ($K = 2.0 \times 10^3$ M^{-1} at $I = 0.1$ M) is well in line with those obtained under low ionic-strength conditions (table 2 of ref. 15). Several previous determinations carried out under different conditions and by using other techniques yielded stability constants which are of a magnitude comparable to that reported here [22].

Metal ion binding to poly(I) is a complicated process. In the presence of the metal ions, Na^+, K^+, etc., poly(I) very slowly forms four-stranded helical complexes in which the metal ions occupy an axial channel where they are coordinated to inosine carbonyl oxygen atoms [14]. Therefore, the present studies were carried out in 0.1 M TMACl. However, the addition of Mg^{2+} and Ni^{2+} also leads gradually to aggregation and finally precipitation. Thus, the stability constants given in this paper can only be considered as approximate values for the binding of Mg^{2+} and Ni^{2+} to (single-stranded?) poly(I). Ni^{2+} binds considerably more strongly to inosine and IMP^{2-} as compared to adenosine and AMP^{2-} [8c]. Therefore the fairly high value of K for Ni^{2+}-poly(I) derived from the ultraviolet data appears plausible. The murexide measurements confirm the strong binding of Ni^{2+} to poly(I). ORD measurements and the melting curves of poly(I) obtained in the presence of divalent metal ions indicate that with Mg^{2+} a multistranded ordered structure is also formed [23]. On the other hand, the base-binding tendency of Ni^{2+} will partially destabilize the ordered structure; but Ni^{2+} may effectively form cross-links between poly(I) strands and thus cause aggregation (and precipitation) [23].

Poly(C) and poly(U) exist under the conditions of the present study predominantly in the single-stranded form [16,23]. Measurements of the ORD and ultraviolet spectra demonstrate that Ni^{2+}, like Mg^{2+}, stabilizes a single-helix conformation of these two polynucleotides [23]. The results indicate that Ni^{2+} binds only to phosphate, not to the bases of poly(U) and poly(C) [23]. This conclusion is consistent with the observation that Mg^{2+} and Ni^{2+} do not noticeably interact with the base in the monomeric cytidine phosphates [24] and UMP

[25]. Moreover, kinetic studies (this paper and ref. 15) indicate that metal ion binding to poly(U) and poly(C) is predominantly nonspecific (i.e., electrostatic; mobile cloud); evidence for site binding could not be detected. Such a binding mode explains at once why the apparent constants for the binding of Ni^{2+} and Mg^{2+} to each of the two polynucleotides are nearly equal (table 2).

The data for the binding of Ni^{2+} to poly(U) and poly(C) obtained by the murexide method are much less informative than those from the ultraviolet data, because of the limited concentration range. The near constancy of $n/[Ni^{2+}_{free}]$ at very low degrees of occupancy (see fig. 4) reveals that the concentration of bound Ni^{2+} is approximately proportional to that of free Ni^{2+}. Within this range (very low n) the ratio $n/[Ni^{2+}_{free}] = [Ni^{2+}_{bound}]/[Ni^{2+}_{free}][poly(N)]_0$ represents an equilibrium constant in terms of monomeric units as binding sites, K'. The value $K' \approx 30$ M^{-1} for Ni^{2+}-poly(U) is equivalent to that determined from ultraviolet spectrophotometry ($K = 67$ M^{-1}) if it is assumed that 2.2 monomeric units form one binding site. Similar considerations apply to Ni^{2+}-poly(C).

It was mentioned already in section 3.1.3 that the ultraviolet difference spectra for metal ion binding to poly(C) show wavelength-dependent differences which cannot be accounted for in terms of a single binding equilibrium as expressed by eq. 1. In other words, these systems cannot be described by a two-state model. The observations can be rationalized, however, by the assumption that with increasing metal ion concentration different conformational states are formed which differ in the shape of their ultraviolet absorption bands. This example reminds us again that the ultraviolet studies do not really measure metal ion binding (therefore: 'apparent' binding constants) but conformational transitions of the polynucleotides.

Although the binding of Ni^{2+} and Mg^{2+} to poly(C) and poly(U) and of Mg^{2+} to poly(A) is dominated by electrostatic interactions (no evidence for site binding), the apparent binding constants at an ionic strength of 0.1 M differ widely, from 65 to 2×10^3 M^{-1}. Obviously, then, these variations have to be assigned to differences in the conformation of the polynucleotides (in the metal-free state) which lead to appreciable differences in the charge density. It is well established that at 25 °C, poly(U) does not possess any order (stacking or helix formation); it is just a structureless, flexible polyelectrolyte [16]. Therefore, its charge density will be low and so are the binding constants. On the other hand, poly(A) above pH 6 is characterized by a high degree of stacking and single-strand helix formation [16]. Clearly, these features produce a rather compact structure of relatively high charge density; therefore, strong electrostatic interactions with metal ions occur. Poly(C) is also known to undergo stacking [16]. For pyrimidine bases the stacking interactions should be weaker than for purine bases. It is therefore plausible that in the case of poly(C) the structural order, charge density and the metal ion binding strength are intermediate between those of poly(U) and poly(A).

The order of the stability constants for binding of Ni^{2+} and Mg^{2+} to single-stranded polynucleotides which has been determined here, poly(A) > poly(C) > poly(U), was also reported (using NMR techniques) for the binding of Mn^{2+} [17]. However, only small differences were found for the binding of Mg^{2+} to these polynucleotides at low ionic strength ($K = 2.1 \times 10^3$–6.3×10^3 M^{-1}) [22a]. In this study, a cation-specific electrode was used in order to determine the concentration of free Mg^{2+} in 0.005–0.02 M solutions of the polynucleotides [22a].

Finally, a few words on the kinetic studies with Ni^{2+}-poly(I) solutions are necessary. At least five reaction time constants have been observed in these solutions (table 1). Thus, a multitude of different poly(I) species must be present, including aggregated species (see section 3.1.4). In view of the many unknown equilibria a detailed analysis of the kinetic data seems impossible. Moreover, very slow processes (up to hours) are known to occur during aggregation reactions of poly(I) [14], and so it might well be that our reactant solutions were not really at equilibrium. Therefore, only some tentative interpretations shall be given: the very fast initial absorbance change (τ_T) is certainly due to the helix-coil transition of single-stranded poly(I); its amplitude decreases with increasing Ni^{2+} concentration because of progressive aggregation. There is a relatively slow effect, τ_4, which appears only at higher Ni^{2+} concentrations. This is most likely a (first-order?) process of aggregated poly(I). The time constant τ_3 decreases with increasing $[Ni^{2+}]_0$, while its amplitude increases. This effect could be due to the binding of Ni^{2+} to aggregated poly(I), or perhaps to the formation of cross-links. The time constants τ_1 and τ_2 are similar to those observed for Ni^{2+}-poly(A) [9], and could be related to the binding of Ni^{2+} to single-stranded poly(I). Such an assignment is also supported by the amplitude variation of τ_1. More definite interpretations cannot be given at this stage. It should be mentioned that in the absence of Ni^{2+} only the fast initial effect is observed (τ_T), but not τ_1–τ_4.

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